J Food Sci Technol (December 2022) 59(12):4695–4705
https://doi.org/10.1007/s13197-022-05551-9

ORIGINAL ARTICLE

Antimicrobial activities of emulsion-based edible solutions incorporating lemon essential oil and sodium caseinate against some food-borne bacteria

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Revised: 8 July 2022 / Accepted: 14 July 2022 / Published online: 28 July 2022

Abstract The antimicrobial properties of two different emulsion formulations contained lemon essential oil (coarse emulsion; CE and nanoemulsion; NE) and emulsions based edible solutions incorporated with lemon essential oil and sodium caseinate (coarse emulsion based solution; CESC and nanoemulsion based solution; NESC) on food-related microorganisms (*Photobacterium damselae*, *Pseudomonas luteola*, *Salmonella* Paratyphi A NCTC13, and *Listeria monocytogenes* ATCC19112) were investigated. The chemical compositions of lemon essential oils were identified by GC–MS. Physical parameter of different formulations was also analyzed at different time intervals. The antimicrobial properties of solutions were determined by using well diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration, and time-kill assay. The major identified component in lemon essential oil was D limonene (38.38%). NE showed a stronger antimicrobial effect against *S.* Paratyphi A and *L. monocytogenes* with 36.50 and 38.75 mm diameter zone compared to all other formulations. *Listeria monocytogenes* had the highest sensitivity towards NE and NESC formulations with 3.12 mg/ml MIC values in comparison to other two formulations. The nanoemulsion and nanoemulsion based coating solution were more effective than other formulations in killing bacterial cell within a short period time.

Keywords Emulsion based solution · Lemon essential oil · Pathogen bacteria · Antimicrobial activity · MIC · MBC

Abbreviations

CE	Coarse emulsion
NE	Nanoemulsion
CESC	Coarse emulsion based solution
NESC	Nanoemulsion based solution
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentrations
PUFA	Polyunsaturated fatty acids
GRAS	Generally considered as safe
SC	Sodium caseinate
PDI	Polydispersity index
GC–MS	Gas chromatography–mass spectrometry
TW	Tween 80
MHB	Muller Hinton broth
MHA	Muller Hinton Agar

Introduction

Fish is valuable source of protein and polyunsaturated fatty acids (PUFA) for human diet, and one of the best animal foods desired by consumers. However, spoilage of fish due to both microbial spoilage and biochemical reaction during processing, storage, and distribution generally causes safety concerns and economic losses. Their high post-mortem pH (> 6), high water activity, and the existence of high level of compounds with low molecular weight in muscles encourage the rapid growth of microorganisms and lipid oxidation, leading to spoilage and short shelf life (Ozogul et al. 2020). Furthermore, microbial contamination may occur from contaminated aquatic environments, diets, cultural

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practice, processing, and distribution of products (Yazgan et al. 2019). Food poisoning resulting from the consumption of fish products contaminated with food-borne pathogens is a significant public health problem worldwide. Some of the major pathogenic bacteria that contaminate fish and meat products are *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus*, *Klebsiella pneumoniae*, *Salmonella* and *Escherichia coli* (Mahmoudzadeh et al. 2017; Degala et al. 2018).

The controlling and monitoring the type and quantity of pathogenic bacteria have become a significant problem for the seafood processing industry. Therefore, various conservation techniques have been performed recently to increase the overall safety of fish and fish products. In recent years, researchers in food industry have focused on the use of natural and broad-spectrum antimicrobial agents for fish and meat products safety with a view to prolonging the shelf life. One convenient option that can be used as a natural antimicrobial agent is the application of the plant based essential oil from various sources such as thyme, rosemary, sage, lemongrass, and cinnamon. Essential oils are well-known natural antimicrobials and are generally considered as safe (GRAS) by the US Food and Drug Administration (Mahmud and Khan 2018). These essential oils consist of mixture of aromatic volatile and nonvolatile components with antimicrobial and antioxidant properties. The lemon essential oil, for example, has been reported to have fungicidal, antioxidant and antimicrobial effects on various species of microorganisms when applied directly in the liquid phase (Arena et al. 2021). Although it shows strong antimicrobial activity, direct application of this essential oil to food has been limited. Essential oils have high volatility, instability and powerful flavor and poor water solubility (Donsi and Ferrari 2016). They can also interact with lipophilic molecules within the food system, for example with proteins and lipids. These interactions may decrease the antimicrobial effect of essential oils. Therefore, suitable approach is necessary to preserve their antimicrobial function, ensure the homogenous distribution of hydrophobic compounds throughout the food matrix, and control release of active compounds, and provide enhanced antimicrobial activity even at a low concentration (Noori et al. 2018; Syed and Sarkar 2018).

Different approaches have been developed for the consolidation of essential oil with different types of delivery vehicles like oil-in-water nanoemulsion or emulsion system, emulsion based edible coating solution including essential oils. Especially, oil-in-water nanoemulsion has been considered as an extremely stable system suitable for delivering vitamins, antimicrobial and antioxidant compounds, and nutraceuticals (Salvia-Trujillo et al. 2017). Nanoemulsions including active compounds might be applied as edible coatings, being a promising strategy to improve the quality, safety, and functionality of solid foods (Acevedo-Fani et al. 2017). It was reported that nanoemulsion based coatings were more efficient than chitosan coating or lemon oil alone (Sessa et al. 2015). Antibacterial activity of modified chitosan based coating solution involving nanoemulsion incorporated with different essential oils (mandarin, lemon essential oil carvacrol, and bergamot) was compared in terms of MIC against *Salmonella* Typhimurium and *Escherichia coli* O157:H7 (Severino et al. 2015). Sodium caseinate (SC) based edible coatings have received considerable attention as a good potential for food applications because of their perfect sensory properties, high nutritional quality, high water vapor, and gas barrier features (Kristo et al. 2008). SC is commercially available at low cost water-soluble polymer obtained from casein which is a major protein in milk (Audic and Chaufer 2005). It also has good film forming capability due to the potential of formation of hydrogen bonding, electrostatic interaction and hydrophobic forces (McHugh and Krochta 1994). Therefore, it is significant to develop efficient nanoemulsion based edible coating solution distribution system to incorporate essential oil and to determine the formulations which will possess better antimicrobial activity against food-borne microorganisms.

The objective of the current study was to compare the antimicrobial activity of two different emulsions (coarse and nano) and emulsions (coarse and nano) based coating solutions incorporated with lemon essential oil and sodium caseinate against fish spoilage (*Photobacterium damselae*, *Pseudomonas luteola*) and pathogen bacteria (*Salmonella* Paratyphi A NCTC13, *Listeria monocytogenes* ATCC19112). The nanoemulsions properties including polydispersity index (PDI), droplet size, thermodynamic stability and surface tension during storage were also evaluated.

Material and method

Chemical compositions of essential oil

The chemical compositions contained in the lemon essential oil were identified by Gas chromatography-mass spectrometry (GC–MS) (Clarus 500, PerkinElmer, Waltham, MA, USA) equipped with PerkinElmerSGE non-polar fused silica capillary column (60 m × 0.25 mm). The oven temperature program was from 60 °C (initially held 10 min) to 250 °C at 4 °C/min, and the final temperature was kept for 10 min. The injector temperature was 220 °C. Helium was used as carrier gas at 1.5 ml/min. The injection volume of the sample was 1 µl of diluted oil in hexane with splitless mode. The electric ionization energy for ion source was 70 eV with the temperature of 200 °C. The range of the scanned mass was m/z 35–425, and the temperature of the interface line was 250 °C. The relative percentage of volatile components

of lemon essential oil were determined by comparing their mass spectra data from NIST-MS and WILEY-MS library.

Preparation of coarse and nano emulsion incorporating lemon essential oil

The coarse and nano emulsion including lemon essential oil were prepared by the methods suggested by Noori et al. (2018) with minor modifications. Briefly, tween 80 with Hydrophile-Lipophile balance (HLB = 15) as non-ionic surfactant and distilled water as aqueous phase were used for the preparation of emulsions. The oil phase for both coarse emulsion and nanoemulsion formulation was prepared from mixture of lemon essential oil (25% v/v) and tween 80 (30% of lemon essential oil). After which to prepare emulsion, the oil phase was gradually added to the water phase with stirring continuously and homogenized at 30,000 rpm for 10 min. by ultra-turrax. The coarse emulsion was then applied to prepare the nanoemulsion, to an CY-500 ultrasonic homogenizer (Optic Ivymen System, Barcelona, Spain). The homogenization parameters were 500 W power and 20 kHz emitted ultrasound frequency for 15 min at 90 µm amplitudes. The size of the sonotrode was 60 mm height and 5.6 mm theta. The sonotrode was symmetrically dipped into the sample to the depth of 15 mm. During the sonication process, the temperature differences between initial coarse emulsion and final nanoemulsion were controlled through the use of ice around the baker (15 °C). To complete the final formulation of coarse emulsion, the coarse emulsion was then homogenized at 30,000 rpm for 15 min. by ultra-turrax. One hundred ml of distilled water was poured into both coarse emulsion and nanoemulsion formulations adjusted to concentration of essential oil and tween 80, and then each formulation was homogenized at 3000 rpm for 10 min in ultra-turrax. The final concentrations of essential oil and tween 80 were 14.92 and 4.47%, respectively. Obtained coarse emulsion and nanoemulsion forms were named as CE and NE. The emulsions were characterized in room temperature.

Preparation of coarse and nano emulsion-based coating solution

The emulsion based coating solution was produced as described by Noori et al. (2018) with minor modifications. The sodium caseinate (4 g) was dissolved in 100 ml of distilled water and stirred at controlled temperature for 30 °C at 11,000 rpm in ultra-turrax for 2.40 h. After that, 1.2 g glycerol (30% wt of sodium caseinate) as plasticizer was added to the sodium caseinate solution. At the next stage, the coarse emulsion and nanoemulsion were poured into coating solution and homogenized at 3000 rpm for 10 min in ultra-turrax and then properties of coating solutions were determined. The final concentrations of essential oil and tween 80 in the coating formulation were 14.92 and 4.47%, respectively. The obtained coarse emulsion and nanoemulsion based coating solutions were named as CESC and NESC. The coating solutions were characterized in room temperature.

Characterization of different formulations

Measurements of physical parameter

The mean particle diameter of droplet, polydispersity index (PDI) and zeta potential (ζ-potential) of the coarse emulsions and nanoemulsion based edible coating solutions as a function of storage time were determined by using Mastersizer 2000 (Malvern, UK). All measurements were performed at 25 °C. Surface tension was determined by using a gonyometer (Attension Theta, Biolin Scientific, Espoo, Finland). Refractive index was determined by an Abbetype refractometer (Schimidt þ Haensch ATR W2, Germany).

Determination of time stability during storage

Long-term stability of all formulations was detected by measuring change in droplet size diameter, polydispersity index, ζ-potential, surface tension at the room temperature (25 °C). For this purpose, all these parameters were recorded on the 1th, 7th and 15th day at 25 °C.

Determination of antimicrobial activity

Test microorganisms

The two fish spoilage bacteria including Gram-negative *Pseudomonas luteola* and *Photobacterium damselae* were isolated from spoiled fish and identified in our previous study (Yazgan et al. 2019). The two food-related pathogen bacteria containing Gram-positive *Listeria monocytogenes* ATCC19112 and Gram-negative *Salmonella* paratyphi A NCTC13 were purchased from Spanish Type Culture Collection, (CECT, Valencia, Spain) and National Collection of Type Cultures, (London, UK), respectively.

Bacterial inhibition assay using the well diffusion method

The bacterial inhibition impact of two different emulsions (coarse and nano) was compared with the emulsions (coarse and nano) based coating solutions incorporated with lemon essential oil and sodium caseinate using agar well diffusion method (Hwanhlem et al. 2017) with minor modifications against two fish spoilage and fish-borne pathogen bacteria including *P.damselae*, *P.luteola* and *S.* Paratyphi A NCTC13 and *L. monocytogenes* ATCC19112. Briefly, overnight

bacterial strains were adjusted to 10^6 cfu/ml. Following this, each bacterial suspension was spread on Muller Hinton Agar (MHA, Merck, Germany) plates. The agar wells of 5 mm in diameter were made in the agar plate using steril plastic cylinder. Each well was filled with 100 µl of the different formulations. As negative control, 100 µl of sodium caseinate (SC), Tween 80 (TW), and Muller Hinton broth (MHB, Merck, Germany) were placed into the wells. They were allowed to diffuse for 30 min. The plates were then incubated at 37 °C for 24 h. After incubation, the inhibition zones around each well were measured and recorded.

Determination of minumum inhibitory and minimum bactericidal concentration (MIC and MBC)

The MIC values were determined using Clinical and Laboratory Standards Institute's (CLSI) methods (2008). One ml of each different formulation (stock solution of 50 mg/ml) was added into the first tube in each series and serially diluted with Mueller Hinton Broth (MHB, Merck, Germany). One ml of each inoculum suspension that included 6 log CFU of each bacterial strain was put into each tube including different formulations and MHB. The final concentration of the formulations were 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 mg/ml. MIC was evaluated as the minimum concentration of different formulaitons needed to prevent visible bacterial growth in tubes after incubation at 37 °C for 24 h. The tube containing SC, tween 80 or MHB and bacterial suspension without formulations was used as control. The tube without MHB was also used as a negative control. MBC was determined according to the CLSI quidelines. Briefly 0.1 ml of clear tube, which did not show bacterial growth after incubation during MIC test was spread on Muller Hinton Agar (MHA) agar plate and incubated at 37 °C for 24 h. The number of bacterial colonies was counted and expressed as log cfu/ml.

Time-kill assay

The time-kill assay of emulsions and emulsion based coating solutions prepared from different formulations against all tested bacteria were investigated at their MIC according to the method of the Chuesiang et al. (2019) with minor modifications. Briefly, 1 ml of overnight growth of each bacterial suspension (10^6 cfu/ml) was inoculated to each tube including all formulations at MIC concentration. The tubes were incubated for 0, 1, 3, 6, 9, 12 and 24 h at 37 °C. 0.1 ml of each bacterial dilution was spread on MHA agar plate at each time point and incubated at 37 °C for 24 h. The tube including bacteria but no different emulsion based formulations acted as control. Bacterial colonies were counted after incubation.

Statistical analysis

Mean value and standard deviation of three samples for each treatment were measured. The significance of differences ($p < 0.05$) was determined using the SPSS Version 20 statistical package (SPSS, Chicago, IL. USA).

Results and discussion

Chemical compositions of essential oil

The variety of chemical compositions of essential oil is important as it affects their antibacterial activity. Thus, it is fairly significant to determine the chemical compounds contained in the essential oil. The major compound in the lemon essential oil identified by GC/MS libraries were D-limonene (38.38%), β-pinene (12.94%), p-cymene (9.76%) and limonene glycol (8.59%) (Fig. 1). The other components in low concentrations were also obtained

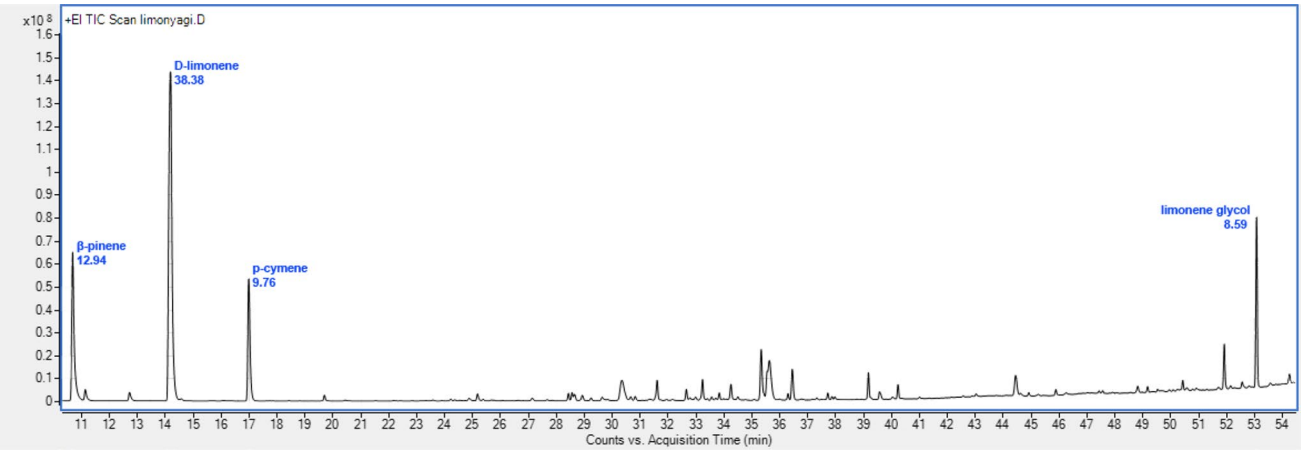

Fig. 1 GC–MS chromatogram for major component of lemon essential oil

from GC/MS libraries. Several authors have reported that the major components found in the lemon essential oil are p-cymene, D-limonene, γ-terpinene, β-pinene, tri-cyclen, limonene (Moosavy et al. 2017; Yazgan et al. 2019). The chemical composition of essential oils differs significantly depending on the season and harvest time, genetic variability of herb, geographical condition, and extraction methods (Marino et al. 2001).

Characterization of different formulations

Measurements of physical parameter

The effect of emulsion formulations and preparation technique on physical parameter like particle size diameter, their distribution in emulsion system (PDI), zeta potential (ζ-potential) and surface tension is presented in Fig. 2. Particle size diameter (Z-average) of emulsion, and PDI (Polydispersity index) in emulsion system play an important role in determining the stability of emulsion and emulsion based products (Syed and Sarkar 2018). Furthermore, they have a considerable effect on the in vivo fate of the nanoemulsion. Thus, it is significant to detect the PDI value and particle size of the emulsion. In the current study, the smallest size diameters were obtained from the NE and NESC (Fig. 2a). Many researchers have manufactured nanoemulsion incorporating essential oils into biopolymer solutions such as chitosan and sodium alginate prior to sonication. All these researchers reported larger droplet diameter (169 -490 nm) (Acevedo-Fani et al. 2015; Wu et al. 2016; Artiga-Artigas et al. 2017) than that of findings obtained in the current study. On the other hand, in the CE and CESC prepared using ultra-turrax, larger particle size diameters with 342.00 and 330.10 nm were observed. These differences between the emulsion and coating solution prepared by these two methods indicate that the ultrasonic homogenizer is favorable for the preparation of oil-in-water-emulsion and emulsion based coating solution. The PDI values were detected as 0.038 and 0.087 for NE and NESC, respectively. There was an increased PDI value in the NESC formulation created by adding sodium caseinate (Fig. 2a). PDI values were increased to 0.362 for CE and 0.370 and CESC, when conventional emulsion was fabricated. The narrow density peak and low PDI value of nanoemulsion and nanoemulsion based coating solution confirmed the efficacy of the ultra-sonication method. All the formulations having PDI value of < 0.5 indicate that the samples have a narrow size distribution and will probably be stable (Zhang et al. 2017). Yazgan et al.

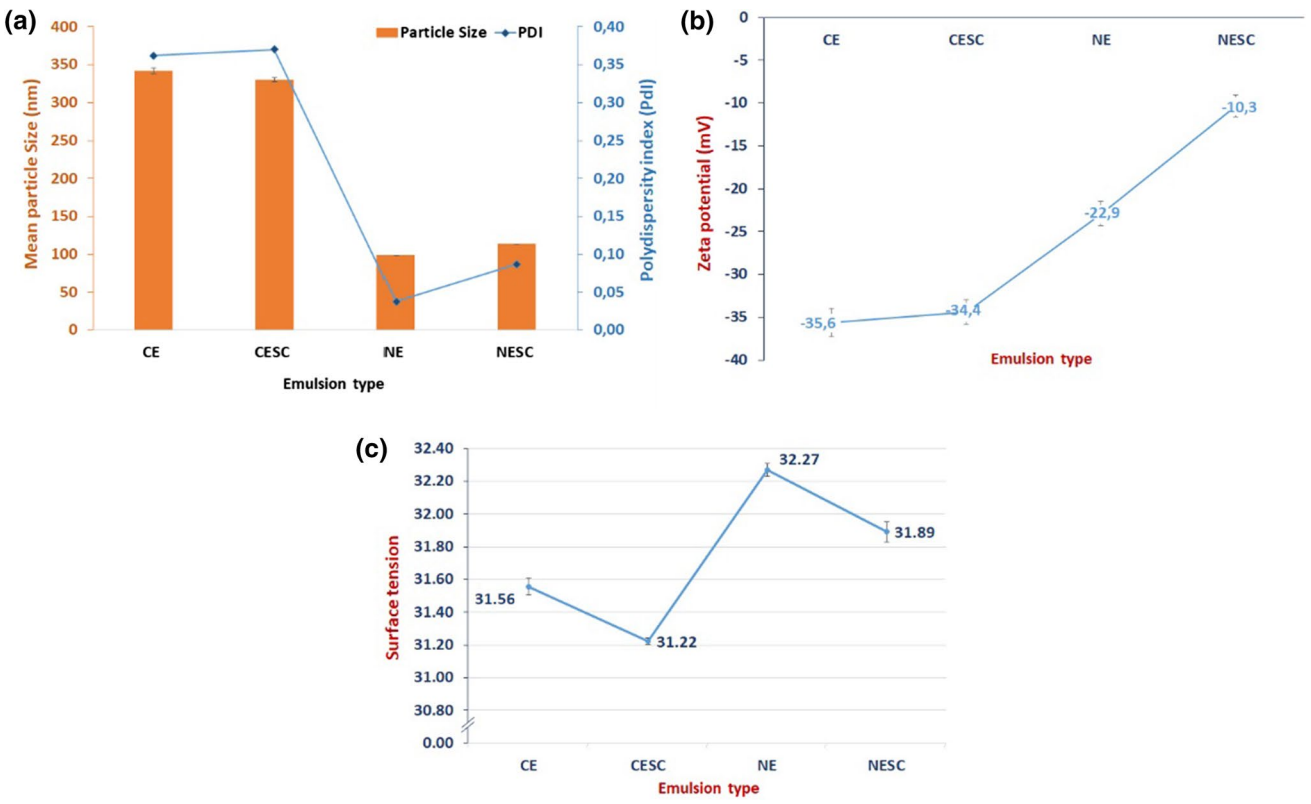

Fig. 2 The effect of emulsion formulations and preparation technique on physical parameter. CE: coarse emulsion, NE: nanoemulsion, CESC: coarse emulsion based solution, NESC: nanoemulsion based solution

(2019) reported larger particle size diameter (181.5 nm) and PDI (0.114) for nanoemulsion containing lemon essential oil prepared by only using an ultrasonic homogenizer. In the other study conducted by Walker et al. (2017), it was reported that the droplet size diameter and PDI of lemon essential oil based nanoemulsion prepared using Tween 80 and a high-pressure homogenizer were 91 nm and < 0.22, respectively. PDI values of the nanoemulsion produced using four different formulations including lemon essential oil were reported in the range of 0.07–0.18 (Donsì et al. 2012). ζ-potential is one of the mostly used parameters used to determine the surface electric charge of oil droplets in emulsion systems. It is also an indicator of emulsion stability (Syed et al. 2020) and can also affect the antimicrobial activity. Nanoemulsions are considered to be stable when the electric charge of the droplets is above + 30 mV or below − 30 mV (Acevedo-Fani et al. 2015). Significant negative ζ-potential production has been reported when non-ionic surfactants are used in the emulsion formulation (Maruno and Rocha-Filho 2009). In the current study, negative charge of oil droplet was recorded in all formulations (Fig. 2b). CE and CESC with the ζ-potential values of − 35.6 and − 34.4 mV showed a strong electrical charge in comparison to NE and NESC. NESC exhibited a relatively weak ζ-potential (− 10.3 mV) in comparison to other formulations. This can be explained by observing a better stability in the conventional method in the formulation containing sodium caseinate. On the other hand, NE showed good stability with the ζ-potential value of − 22.3 mV. The surface tension was recorded at values ranging from 31.22 to 32.27 for all emulsion formulations (Fig. 2c). The technique used to prepare the mixture significantly influenced the emulsion and emulsion based coating solution.

Determination of time-stability during storage

Various techniques can be used to measure time-stability and sustainability in emulsion systems, among which physical parameters such as droplet size diameter, PDI index, and ζ-potential measurement time are more reliable. In the current study, the effects of storage time on physical parameter for all formulations stored at room temperatures were measured on the 1th, 7th and 15 th day. The changes in droplet size diameter, PDI index, ζ-potential and surface tension are given in Fig. 3. The droplet size diameter of all the formulations increased rapidly during the initial 7 days of storage

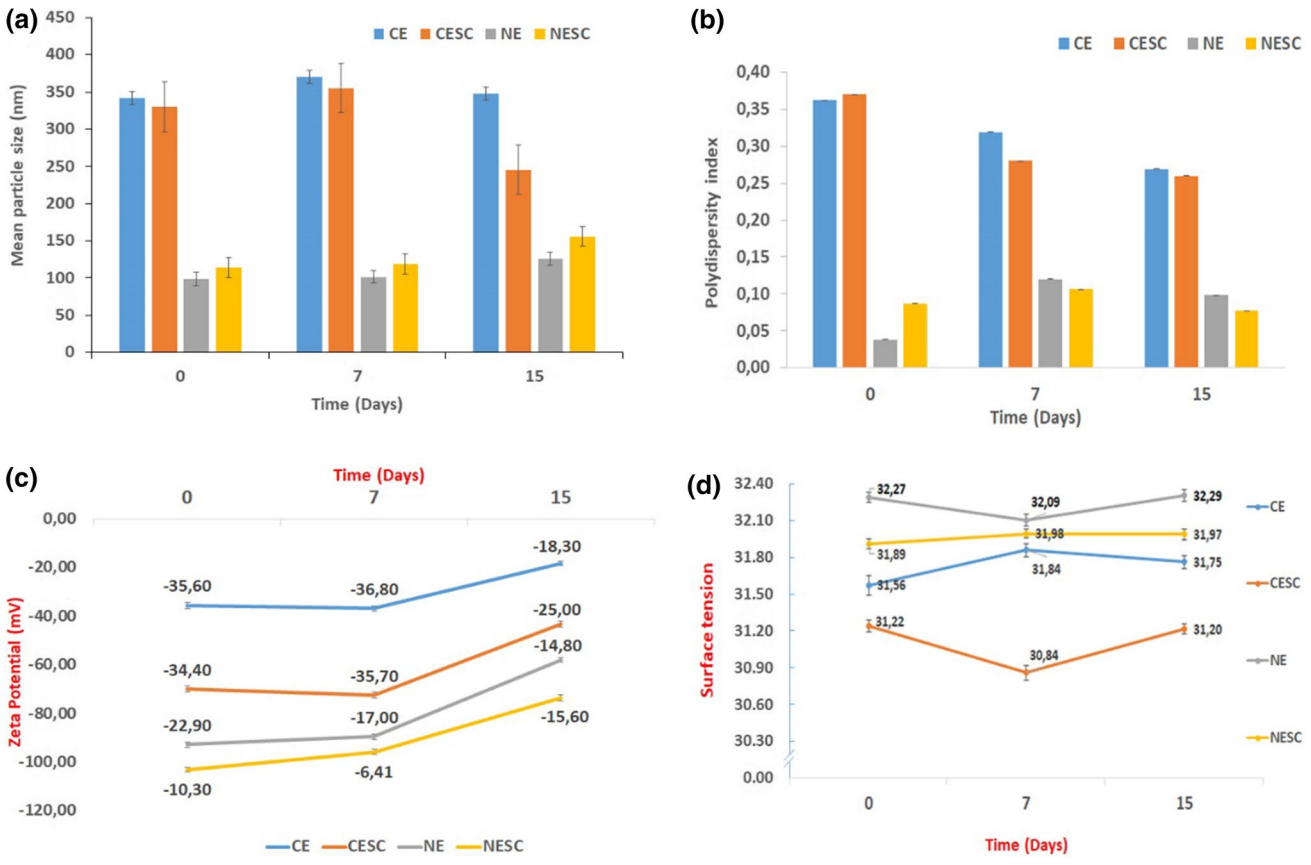

Fig. 3 Polydispersity index (PDI), droplet size, zeta potential (ζ-potential) and surface tension of different formulations at different time intervals. CE: coarse emulsion, NE: nanoemulsion, CESC: coarse emulsion based solution, NESC: nanoemulsion based solution

at room temperatures. However, at the end of the 7th day until day 15th, a decrease in the droplet size diameter for CE and CESC was observed, while the droplet size diameter for NE and NESC continued to increase more slowly (Fig. 3a). However, all formulations incorporating essential oil and sodium caseinate exhibited droplet size still in nano-scale range. This result can be explained as follow, during the droplet formation in oil-in-water emulsion system, the newly formed droplets gains more energy from the applied force, therefore they need time to reach thermodynamic stability (Lu et al. 2018). The PDI values decreased in CE and CESC samples over time in parallel with the change in droplet size (Fig. 3b). A very slow increase was observed in the PDI values of NE and NESC in parallel with the slow increase in droplet diameter as expected. During 15 days of storage, a very strong electric charge was observed with a negative ζ-potential value of more than −30 mV in the first 7 days in CE and CESC samples obtained by the conventional method, but a reduction in ζ-potential values was observed after the 7th day (Fig. 3c). While a continuous decrease was observed in ζ-potential values of NE emulsion over time, a decrease was observed in NESC emulsion within 7 days and an increase was observed after the 7th day. The impact of surface tension is to minimize surface area, resulting in curved surface as in a vesicle. The intensity of this tension depends on the liquid, solvent purity, and temperature in which the surfactant lies (Barradas and Silva 2020). Therefore, similar results were observed over time in surface tension for all formulations (Fig. 3d).

Determination of antimicrobial activity

Bacterial inhibition assay using the well diffusion method

The findings obtained from the well diffusion method against the food-borne bacteria are presented in Table 1. All the formulations exhibited a strong antimicrobial activity against Gram-negative *S.* Paratyphi A NCTC13. However, NE statistically showed a stronger inhibitory effect against the same bacteria with 36.50 mm diameter zone ($p < 0.05$) than the other formulations. In general, Gram-negative bacteria are mostly less sensitive to essential oils, due to the complex structure of their cell walls (Seow et al. 2014), yet, such a trend was not observed in the current investigation. Gram positive *L. monocytogenes* ATCC19112 was more sensitive to the NE with a 38.75 mm diameter zone compared to all other formulations. On the other hand, CE and CESC formulations showed statistically a similar inhibitory impact ($p > 0.05$) against *L. monocytogenes* with the inhibition zone diameter of 26.50 and 26.00 mm, respectively. Das et al. (2020) evaluated the antimicrobial effect of sodium alginate-based edible coating including nanoemulsion of *Citrus sinensis* essential oil on *S.* Paratyphi and *L. monocytogenes* and reported inhibition zone in the range of 8–14 mm. It has been reported that nanoemulsion based on lemon essential oil prepared by ultrasonic method and having larger droplet size exhibited lower antimicrobial activity against the *S.* Paratyphi A (Yazgan et al. 2019) than the result obtained in this study.

NE and NESC were found to be highly effective on fish spoilage bacteria Gram-negative *P. damselae* with 33.25 mm and 32.00 mm inhibition zone values. However, it was observed that Gram-negative *P. luteola* was less sensitive to the CE and CESC compared to the NE and NESC (Table 1). A different result was also obtained by Yazgan (2020) who reported that nanoemulsion incorporated lemon essential oil has an inhibitory effect on *P. damselae* and *P. luteola* with the zone diameter of 19.25 and 17.25 mm, respectively. It was indicated that the inhibitory effectiveness of emulsion or nanoemulsion incorporating essential oil is related to essential oil composition, bacteria strains and nanoemulsion or emulsion formulations and their size (Donsi and Ferrari

Table 1 The inhibition zone diameter (mm) of two different coarse and nano emulsions based solution on fish spoilage and food-borne pathogen bacteria

Treatment groups	Food-borne pathogen bacteria		Fish spoilage bacteria	
	Salmonella Paratyphi A	*Listeria monocytogenes*	*Photobacterium damselae*	*Pseudomonas luteola*
CE	30.00 ± 0.82^{b}	26.50 ± 0.58^{c}	29.25 ± 0.50^{b}	8.50 ± 0.58^{c}
CESC	24.25 ± 0.96^{d}	26.00 ± 0.71^{c}	25.00 ± 0.82^{c}	4.00 ± 0.82^{d}
NE	36.50 ± 0.58^{a}	38.75 ± 0.50^{a}	33.25 ± 0.65^{a}	22.50 ± 0.58^{a}
NESC	25.25 ± 0.50^{c}	35.25 ± 0.96^{b}	32.00 ± 0.41^{a}	12.75 ± 0.65^{b}
TW	0.00 ± 00^{e}	0.00 ± 00^{d}	0.00 ± 00^{d}	0.00 ± 0^{e}
SC	0.00 ± 00^{e}	0.00 ± 00^{d}	0.00 ± 00^{d}	0.00 ± 0^{e}

CE coarse emulsion, *NE* nanoemulsion, *CESC* coarse emulsion based solution, *NESC* nanoemulsion based solution, *TW* Tween 80, *SC* sodium caseinate

Values represents mean ± standard deviation. The same superscript (a-e) in the same row were not significantly different ($P > 0.05$)

2016). The antimicrobial effects of pure SC solution and Tween 80 against all tested bacteria were not observed.

Determination of MIC and MBC

MBC of CE and CESC formulations against all bacteria tested was 50 mg/ml (Table 2). The MIC value of CE against fish spoilage and pathogen bacteria was 25 mg/ml for *L. monocytogenes* and 50 mg/ml for *S.* Paratyphi A, *P. damselae* and *P. luteola*, whilst the MIC value of CESC was 50 mg/ml for all the bacteria. Among the all bacteria tested, *L. monocytogenes* had the highest sensitivity since the lower concentration of NE and NESC formulations was required with 3.12 mg/ml MIC values in comparison to other two formulations (Table 2). The bactericidal effect was also observed on *L. monocytogenes* with MBC value of 25 mg/ml for NE and NESC. *Salmonella* Paratyphi A had the low MIC value (12.50 mg/ml) for NE and NESC formulations. These results are consistent with our previous work on lemon essential oil based nanoemulsion, which showed the similar inhibition properties against *S.* Paratyphi A (Yazgan et al. 2019). The bacterial growth of *P. damselae* was also inhibited by NE and NESC formulations with MIC values of 6.25 and 12.50 mg/ml, respectively. Similarly, NESC formulations showed good bactericidal impact on the same bacteria with MBC values of 12.50 mg/ml. The growth of all the bacteria in the control treatment with sodium caseinate and tween 80 was similar indicating that they have no antimicrobial activity.

Time-kill assay

The time-killing assay was carried out to determine the change of viability of all bacteria upon interaction with all formulations during 24 h period. There were significant decreases in bacterial cells on exposure to MIC concentration of all formulations (Fig. 4). *S.* Paratyphi A was the most sensitive to all treatments since it was quickly inhibited at the fastest rate within 30 min. and 1 h after being treated with CE, NE, CESC and NESC formulations, respectively (Fig. 4a). This result was in agreement with the previous reports that showed nanoemulsions were able to kill bacterial cells within a short period time (Moghimi et al. 2016; Prakash et al. 2019). *L. monocytogenes* was also inhibited after 1 h contact with CE and NE formulations whereas the CESC and NESC formulations required a longer contact time (3 h) for inhibition. On the other hand, whilst NE formulation treatment prepared by ultra-sonication exhibited an approximately 4 log reduction in the cell number of *L. monocytogenes* within 30 min, CE formulation exerted 2 log reduction in the cell number (Fig. 4b). A possible explanation is that NE formulation has the lemon essential oil in a form of small droplet size and thus the small essential oil droplets are able to easily pass through the outer membrane of the bacterial cell. Several authors reported that nanoemulsion incorporated with essential oil prepared by ultra-sonication was more effective on several food-borne pathogen bacteria (Topuz et al. 2016; Moghimi et al. 2016). The *P. damselae* treatment with CESC and NESC formulations for 3 h resulted in approximately 3 log cfu/ml reduction in viable cells and a complete loss of viability in 6 h (Fig. 4c). On the other hand, the viable cells of this bacteria were killed within 30 min and 1 h of exposure to NE and CE formulations, respectively. The *P. luteola* was also reduced at detectable levels within 9 h of interact with CE and CESC formulations and loss of viability in 12 h (Fig. 4d).

Conclusion

The study findings clearly revealed that the coarse emulsion based coating solution with sodium caseinate showed lower inhibitory effect against all the bacteria tested. In addition, it has been observed that the nanoemulsion and nanoemulsion based coating solution obtained by the ultrasonic methods have a stronger inhibition effect against all

Table 2 MIC and MBC determination of two different coarse and nano emulsions based solution on tested microorganisms

Treatment groups	Food-borne pathogen bacteria				Fish spoilage bacteria			
	Salmonella Paratyphi A		*Listeria monocytogenes*		*Photobacterium damselae*		*Pseudomonas luteola*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MB (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
CE	50	50	25	50	50	50	50	50
CESC	50	50	50	50	50	50	50	50
NE	12.50	50	3.12	25	6.25	50	25	50
NESC	12.50	50	3.12	25	12.50	12.5	25	50
TW	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
SC	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50

CE coarse emulsion, *NE* nanoemulsion, *CESC* coarse emulsion based solutions, *NESC* nanoemulsion based solutions, *TW* Tween 80, *SC* sodium caseinate

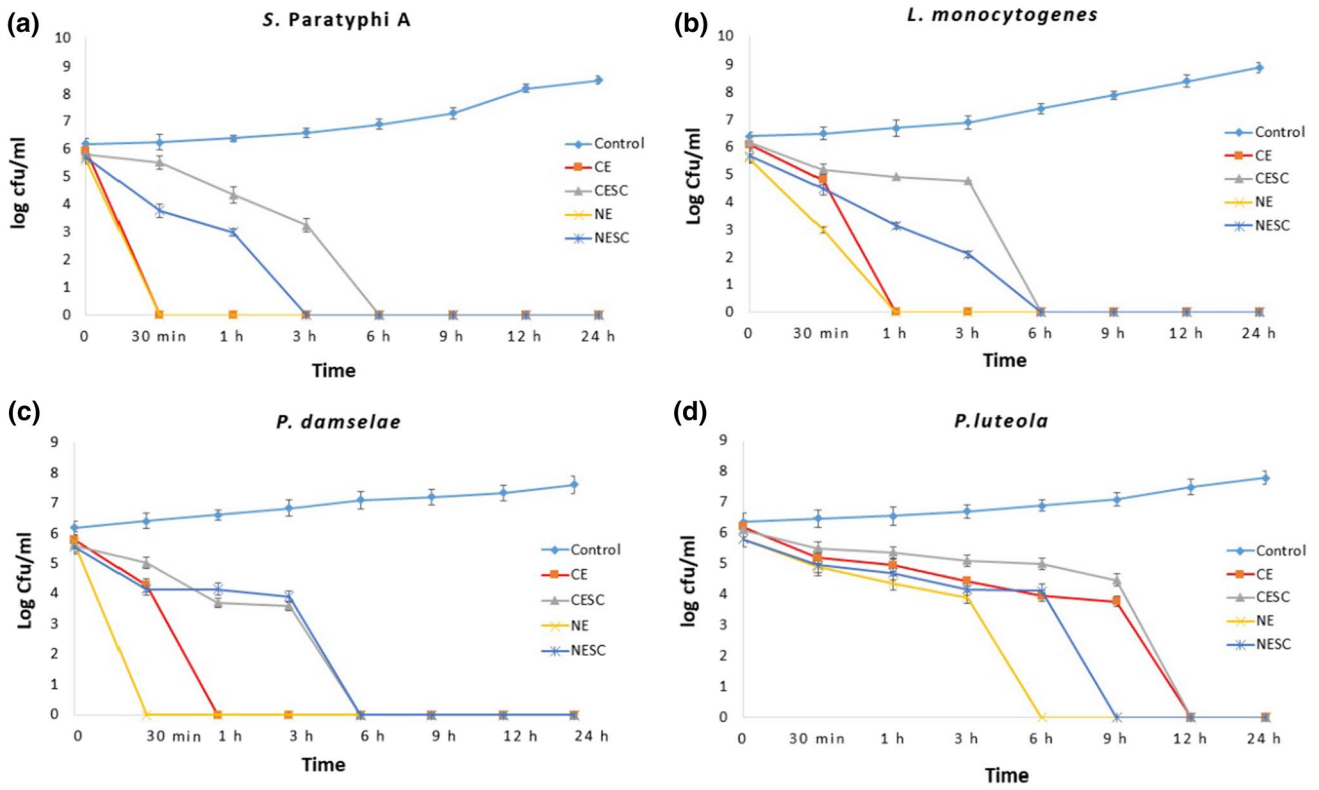

Fig. 4 Time-kill curves of *Salmonella* Paratyphi A, *Listeria monocytogenes*, *Photobacterium damselae*, *Pseudomonas luteola* treated with two different emulsion and emulsion based edible coating material. CE: coarse emulsion, NE: nanoemulsion, CESC: coarse emulsion based coating solution, NESC: nanoemulsion based coating solution

the bacteria tested when compared to the conventional method. Nanoemulsion and nanoemulsion based edible coating solutions incorporated with lemon essential oil and sodium caseinate obtained by ultra-sonication method exhibited an enhanced and faster bactericidal and inhibitory effect against all the bacteria tested in comparison with the coarse emulsion and emulsion based on edible coating solution. The results of time killing assay show that nanoemulsion and nanoemulsion based coating solution obtained by the ultrasonic methods was more effective than the other formulation in killing the bacterial cells within a short period time. The study results further revealed that nanoemulsions prepared by different formulations were more effective than the course emulsion and that they had a potential application as antimicrobial in food industry.

Acknowledgements The author thanks the Scientific Research Projects Department of Cukurova University, Adana, Turkey.

Author contributions HY: Conceptualization, original draft, validation, investigation, statistical analysis, writing, and editing.

Funding This research was financially supported by Scientific Research Projects Department of Cukurova University, Adana, Turkey. The author thanks Department of Scientific Research Projects (BAP) for their support.

Availability of data and material The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The author declares no competing interest. Ethics approval was not required for this research.

Ethical approval Ethics approval was not required for this research.

Consent to participate Not applicable.

Consent for publication Not applicable.

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